Volume 330, number 1, 77–80 FEBS 12937 September 1993

cDNA cloning and pharmacological characterization of an opioid receptor with high affinities for κ-subtype-selective ligands

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Received 19 July 1993

The amino acid sequence of a rat opioid receptor, designated as ROR-D, has been deduced by cloning and sequencing the cDNA. The ROR-D expressed from the cDNA exhibits high affinities for ligands selective for the opioid receptor κ-subtype and low affinities for ligands selective for the δ- and μ-subtypes RNA blot hybridization analysis indicated that ROR-D mRNA is distributed in cerebrum and brainstem but not in cerebellum.

Opioid receptor; cDNA cloning; cDNA expression; RNA blot hybridization analysis, Rat brain

1. INTRODUCTION

Opioid analgesics produce their pharmacological actions by interacting specifically with opioid receptors in the nervous system [1]. The opioid receptors mediate cellular inhibitory effects, including inhibition of adenylate cyclase, activation of potassium channels and inhibition of calcium channels, through the action of guanine nucleotide-binding regulatory proteins (G-proteins) [2,3]. Pharmacological studies have defined at least three distinguishable subtypes (μ, κ and δ) of the opioid receptors on the basis of their difference in affinities for opioid ligands [1,2].

Recently, the cDNA cloning of the δ- and the μ-subtype opioid receptor has been reported and pharmacological properties of the receptors expressed from the cDNAs have been studied [4–6]. In this report, we have cloned cDNA encoding an opioid receptor subtype, designated as ROR-D, from rat cerebrum cDNA libraries, and studied its ligand-binding properties by expression of the cDNA in COS cells. The results showed that the pharmacological properties of the expressed ROR-D correspond most likely to those of the κ-opioid receptor subtype.

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Abbreviations: G-proteins, guanine nucleotide-binding regulatory proteins; EKC, ethylketocyclazocine; DADLE, [D-Ala^2,D-Leu^5]-enkephalin; DPDPE, [D-penicillamine^2,D-penicillamine^5]enkephalin; DAGO, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin.

2. MATERIALS AND METHODS

2.1. *Cloning of cDNA*

Rat cerebrum cDNA libraries [6] were screened with the ~1.4-kilobase pairs (kb) *Hin*dIII fragment derived from pROR15 [6], or an equimolar mixture of the 0.54-kb and 0.52-kb *Sma*I fragments derived from pROR1 [6], as probes. cDNA inserts from clones positive to both probes were subcloned into pBluescript SK(−) and further analyzed. cDNA inserts of 3 clones, including pROR201 and pROR302, share a common restriction endonuclease map (*Pst*I, *Hin*dIII, *Xho*I, *Spe*I, *Xba*I and *Eco*RI), which differs from those of the ROR-A and ROR-B cDNAs [6], and are indicated to be derived from identical mRNAs, designated as ROR-D. The cDNA clones, pROR201 and pROR302, carrying the entire protein-coding sequence, were used for sequence analysis [7,8].

2.2. *Expression of cDNA and ligand-binding assay*

The 0.38-kb *Pst*I–*Eco*RI and 0.78-kb *Eco*RI–*Spe*I fragments from pROR201 were ligated with the *Pst*I–*Spe*I fragment from pBluescript SK(−) to yield pRORS201-1. pRORS201-1 was digested with *Xba*I, blunt-ended with T_4 DNA polymerase, and then ligated with the synthetic *Hin*dIII linker to yield pRORS201-2. pRORS201-2 was partially digested with *Hin*dIII, and the resulting 1.2-kb fragment carrying the entire protein-coding sequence of ROR-D was cloned into the *Hin*dIII site of an expression vector, pKCRH2 [9], in the same orientation with respect to SV40 early promoter to generate pRORS201-4. COS-7 cells were transfected [10] with pRORS201-4 and harvested after 2–3 days. Crude membranes were prepared and assayed for [^{3}H]EKC binding as in [6], except that reactions were performed in 0.2 ml using 20 μg of membrane preparations.

2.3. *RNA blot hybridization analysis*

Poly(A)$^+$ RNAs prepared from adult Wistar rats were analyzed as in [6]. The hybridization probe used was the ~2.3-kb cDNA insert of pROR201. The probe was labelled with [α-^{32}P]dCTP by the random primer method [11]. Autoradiography was performed at −80°C for 3 days with an intensifying screen. An RNA ladder (Bethesda Research Laboratories) was used as size markers.

3. RESULTS AND DISCUSSION

Rat cerebrum cDNA libraries were screened by hybridization with probes derived from the rat δ- and μ-opioid receptor cDNAs, and a novel class of cDNA clones (ROR-D) was isolated (see section 2.1). Nucleotide sequence analysis of the cDNAs reveals an open reading frame of 1,140 nucleotides encoding a sequence of 380 amino acids. Fig. 1 shows the deduced amino acid sequence of the ROR-D protein aligned with those of ROR-A (the δ-opioid receptor subtype) and ROR-B (the μ-subtype). Amino acid sequence comparison reveals 59% and 58% identities between ROR-D/ROR-A and ROR-D/ROR-B pairs, respectively. The ROR-D protein shares characteristic structural features with G-protein-coupled receptors [12]. Seven transmembrane segments are predicted in the ROR-D molecule, and two consensus N-glycosylation sites [13] occur in the amino-terminal region preceding the predicted transmembrane segment, TM-I. In the ROR-D, ROR-A and ROR-B molecules, the carboxy-terminal region just following TM-VII contains two conserved cysteine residues that may be modified by palmitoylation [12]. The cytoplasmic portion between TM-V and TM-VI, which has been suggested to determine the selectivity of G-protein coupling [14,15], is conserved well among these molecules, thus they may couple with similar G-proteins.

An expression plasmid (pRORS201-4) was constructed in which the entire protein-coding sequence of ROR-D is positioned downstream of the SV40 promoter, as in section 2.2. Fig. 2 shows that membrane preparations from COS cells transfected with pRORS201-4 were capable of binding [^{3}H]EKC, a high-affinity ligand for opioid receptor subtypes, whereas negligible binding was found for non-transfected COS cells. The dissociation constant (K) for EKC was estimated by Scatchard analysis to be 0.48 nM (mean of two experiments). Ligand-binding properties of the expressed ROR-D were further examined in comparison with those of ROR-A and ROR-B. The apparent K_d values for representative opioid ligands are listed in Table I. ROR-D showed lower binding affinities for peptide agonists selective for the δ- and μ-opioid receptor subtypes (DADLE with a high affinity for the δ- and μ-subtypes, DPDPE selective for the δ-subtype, and DAGO selective for the μ-subtype) than ROR-A and ROR-B. Morphine and naloxone had moderate affinities for ROR-D among these receptor subtypes. On the other hand, ROR-D exhibited much higher affinities for κ-subtype-selective ligands (U50488, U69593 and EKC) than ROR-A and ROR-B. These results indicated that ROR-D corresponds most closely to the pharmacologically defined κ-opioid receptor subtype. Recent studies have suggested that the κ-opioid receptor subtype can be further classified into the κ_1-type (with a high affinity for U69593) and the κ_2-type (with a low affinity) [16,17]. ROR-D exhibited a K_d = 30 nM for U69593 and may, therefore, belong to the κ_1-type.

The affinity profile of dynorphin(1–13) for cloned opioid receptor species was not consistent with previous observations using brain microsomal preparations. Many experiments have shown that dynorphin(1–13) exhibits the highest affinity for the κ-subtype among

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ROR-A  MEPV-------PSARAELQFSLLANVSDTFPSA- -----FPSASANASGSPG----------------------ARSA SSLALAIAITA  54
ROR-B  MDSS---------------TGP-GNTSDCSDPLAQASCSPAPGSWLNLSHVDGNQSDPCGLNRTGLGGNDSLCPQTGS-PSMVTAITIMA  73
ROR-D  MESPIQIFRGEPGPTCAPSACLLPNSSSWFPN-------WAESDSNGSVGSEDQQ  -------- -------LEPAHISPAIPVLITA  64

ROR-A  LYSAVCAVGLLGNVLVMFGIVRYTKLKTATNIYIFNLALADALATSTLPFQSAKYLMETWPFGELLCKAVLSIDYYNMFTSIFTLTMMSV 144
ROR-B  LYSIVCVVGLFGNFLVMYVIVRYTKMKTATNIYIFNLALADALATSTLPFQSVNYLMGTWPFGTILCKIVISIDYYNMFTSIFTLCTMSV 163
ROR-D  VYSVVFVVGLVGNSLVMFVIIRYTKMKTATNIYIFNLALADALVTTTMPFQSAVYLMNSWPFGDVLCKIVISIDYYNMFTSIFTLTMMSV 154
           TM-I                                TM-II                                TM-III

ROR-A  DRYIAVCHPVKALDFRTPAKAKLINICIWVLASGVGVPIMVMAVTQPRDGA--VVCTLQFPSPSW YWDTVTKICVFLFAFVVPILIITV 231
ROR-B  DRYIAVCHPVKALDFFIPPNAKIVNVCNWILSSAIGLPVMFMATTKYRQGS--IDCTLTFSHPTW YWENLLKICVFIFAFIMPVLIITV 250
ROR-D  DRYIAVCHPVKALDFRTPLKAKIINICIWLLASSVGISAIVLGGTKVREDVDVIECSLQFPDDEYSWWDLFMKICVFVFAFVIPVLIIV 244
                                   TM-IV                                TM-V

ROR-A  CYGLMLLRLRSVRLLSGSKEKDRSLRRITRMVLVVVGAFVVCWAPIHIFVIVWTLVDINRRDPLVVAALHLCIALGYANSSLNPVLYAFL 321
ROR-B  CYGLMILRLKSVRMLSGSKEKDRNLRRITRMVLVVVAVFIVCWTPIHIYVIIKALITIPET-TFQTVSWHFCIALGYTNSCLNPVLYAFL 339
ROR-D  CYTLMILRLKSVRLLSGSREKDRNLRRITRLVLVVVAVFIICWTPIHIFILVEALGSTSHS-TAVLSSYYFCIALGYTNSSLNPVLYAFL 333
                                   TM-VI                                TM-VII

ROR-A  DENFKRCFRQLCRAPCGGQEPGSLRRPPQATARERV-TACTPSDGPGGGAAA          372
ROR-B  DENFKRCFREFCIPTSSTIEQQNSTRVRQNT-REHPSTANTVDRTNHQLENLEAETAPLP 398
ROR-D  DENFKRCFRDFCFPI-------KMRMERQSTNRVRII-TVQDPASMRDVGGMNKPV     380
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Fig. 1. Deduced amino acid sequence of ROR-D (bottom) and its alignment with those of the δ-opioid receptor, ROR-A (top), and the μ-opioid receptor, ROR-B (middle). Sequence data for ROR-A and ROR-B have been taken from [6]. The one-letter amino acid notation is used. Gaps (-) have been inserted to achieve maximum homology. Amino acid residues are numbered from the initiating methionine and numbers of the residues at the right-hand end of the individual lines are given. The predicted transmembrane segments (TM-I–VII) are indicated; the termini of each segment are tentatively assigned. The nucleotide sequence of ROR-D will appear in the DDBJ, EMBL, GenBank Nucleotide Sequence Databases under accession number D16534.

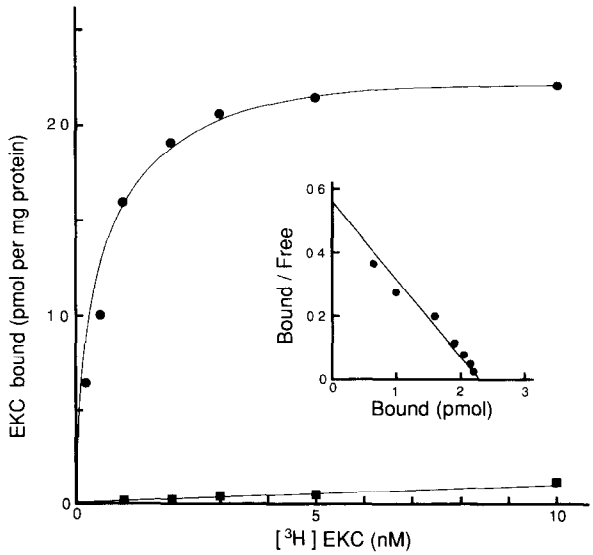

Fig. 2. Binding of [^{3}H]EKC to the ROR-D species expressed in COS cells. Membrane preparations from pRORS201-4-transfected COS cells (●) and non-transfected COS cells (■) were analyzed. The inset shows Scatchard plots of the data. The non-specific binding (measured in the presence of excess amount of unlabelled naloxone) was less than 16% of the total radioactivity bound.

opioid receptor subtypes in the brain [18,19]. In contrast, dynorphin(1–13) showed the lowest affinity for ROR-D in our results (Table I). This discrepancy might suggest that other κ-subtype receptors with higher affinities for dynorphin than that of ROR-D exist in the brain, and/or that brain-specific modifications of the ROR-D molecule are required for a high affinity for dynorphin.

Table I

Ligand-binding properties of defined opioid receptor species expressed from cDNAs

Ligands	Apparent K_d (nM)		
	ROR-D	ROR-A	ROR-B
DADLE	>10,000	5.0	7.9
DPDPE	>10,000	28	1,900
DSLET	ND	12	142
DAGO	3,000	2,240	4.0
Morphine	300	1,260	5.3
Naloxone	15	67	1.8
EKC	0.48	3.3*	0.93*
U50488	12	>10,000	710
U69593	30	3,400*	3,100*
Dynorphin(1–13)	210	53$^+$	40$^+$

The K_d values of ROR-D for EKC were obtained by Scatchard analysis and those for the other ligands were obtained by measuring displacement of [^{3}H]EKC binding; means of at least two experiments are given. The K_d values of ROR-A and ROR-B have been taken from [6] and unpublished experiments using [^{3}H]EKC (marked by asterisks) or [^{3}H]DADLE (marked by plus signs). ND, not determined.

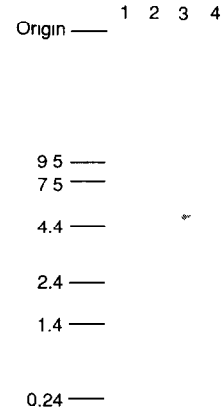

Fig. 3. Autoradiograms of blot hybridization analysis of poly(A)$^+$ RNA from rat tissues with the probe from ROR-D cDNA. Samples of poly(A)$^+$ RNA (30 μg each) prepared from kidney (lane 1), cerebellum (lane 2), brainstem (lane 3) and cerebrum (lane 4) were analyzed as in section 2.3. The size markers are indicated in kb.

Poly(A)$^+$ RNA preparations from rat tissues were subjected to blot hybridization analysis (Fig. 3). Cerebrum and brainstem contained an RNA species of ~5.2 kb hybridizable with the probe derived from the ROR-D cDNA. No hybridizable RNA species was detected in cerebellum and kidney. This result is consistent with previous observations by autoradiographic experiments using radioligands [20], which showed that the κ-opioid receptor subtype is distributed throughout the rat central nervous system except cerebellum. We have previously shown that cerebrum and brainstem also contain ROR-A and ROR-B mRNAs. Thus, the opioid receptor heterogeneity in the nervous system with respect to ligand binding is attributable to various combinations of distinct opioid receptor gene products, including ROR-D, ROR-A and ROR-B.

Acknowledgements We thank Dr. Takashi Miyata and Mr. Naoyuki Iwabe for computer analysis. This work was supported in part by a grant from Shimadzu Corporation.

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